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SAMPLE SELECTION AND TESTING

OF SEPARATION PROCESSSES

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Huntsville Hospital
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FINAL REPORT

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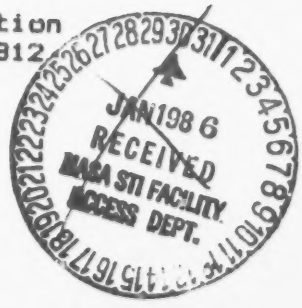

December 1985

INTRODUCTION

The focus of the work completed under this contract has been in the area of phase partitioning, which has become an important tool for the separation and purification of biological materials. Instruments available for this technique were researched and a countercurrent distribution apparatus, the Biosheff MK2N, was purchased from the Biochemistry Department of the University of Sheffield, Sheffield, England, and delivered to NASA. Various proteins, polysaccharides and cells were studied as models to determine operating procedures and conditions for this piece of equipment. Results were compared with those obtained from other similar equipment, including a nonsynchronous coil planet centrifuge device built by Y. Ito and lent to NASA by NIH. Additionally, work was done with affinity ligands attached to PEG, which can further enhance the separation capabilities of phase partitioning.

The most important aspects of the work performed during this contract period have been compiled into two manuscripts (attached as an appendix to this report), one of which has been published in the Journal of Liquid Chromatography. The second manuscript has been accepted for publication in the Journal of Chromatography. Also included is an abstract of a presentation delivered in Lund, Sweden at the 4th International Conference on Partitioning in Aqueous Two-Phase Systems in August, 1985.

BACKGROUND

When mixed in low concentrations, aqueous solutions of the polymers poly(ethylene glycol) (PEG) and dextran form a two-phase system with a PEG-rich upper phase floating on a denser, dextran-rich lower phase. With the addition of salts and buffers, these solutions can be made isotonic and have become important in both analytical and preparative separations of many types of biological substances (see Appendix for references). These separations occur because of differential partitioning between the upper and lower phases, or between one of the phases and the interface, for particulate substances.

Factors which influence the partition of particles or macromolecules include size, surface charge, surface hydrophobicity and system characteristics such as interfacial tension and salt composition. Separations can sometimes be enhanced through the use of affinity ligands, specific for the material of interest, which have been chemically attached to one of the polymers (typically PEG) and, in effect, pull the target substance into the phase rich in that polymer.

When differences in the partition coefficient K (the ratio of the number of cells in the top phase to the remainder of cells) are great enough, samples can be separated in a single tube. However, in most cases, differences are not that large and subsequent extractions are necessary, using automated devices such as a countercurrent distribution (CCD) apparatus.

MATERIALS AND METHODS

Materials and methods used for the experiments described below can be found in detail in the Appendix.

RESULTS

Results obtained using the Biosheff MK2N thin-layer CCD apparatus were compared with a nonsynchronous coil planet centrifuge (NSCPC) designed by Y. Ito for countercurrent chromatography, and two other thin-layer CCD devices; one automated, designed by P.A. Albertsson, Lund, Sweden, and the other a manual model designed by J.A. Van Alstine, Huntsville, Alabama.

All the thin-layer CCD devices gave similar results, with partitioning profiles of trypan blue dye, proteins and various species of red blood cells (both fixed and fresh) being in close agreement with calculated theoretical curves. Theoretical predictions for separation with the NSCPC apparatus are considerably more complicated. However, comparison of partitioning profiles of cells, in systems yielding similar partition coefficients, showed a less than efficient mixing of phases in the NSCPC device. with one run being comparable to twenty to thirty transfers in the CCD devices. Comments regarding advantages and disadvantages of the two kinds of

systems, as well as appropriate references can be found in the Appendix.

Studies performed with affinity ligands to determine their ability to refine separation capabilities of phase partitioning have included using specific ligands (antibodies), to which PEG is covalently attached, to cause substantial changes in the partition coefficient of a particular cell type. As models, rabbit antibody directed against human red blood cells (HRBC) and sheep antibody, also directed against HRBC, were used. PEG molecules were covalently coupled to a portion of the lysine residues of the antibodies, in the laboratory of Dr. J. Milton Harris, University of Alabama in Huntsville, Huntsville, Alabama. The PEG-modified antibodies were incubated with both HRBC and sheep red blood cells (SRBC) causing a dramatic shift in the partition coefficient of the HRBC but no difference in that of the SRBC.

As well as increasing its affinity for the PEG-rich upper phase, the modification of the antibody with PEG decreases its tendency to agglutinate HRBC. These effects can be varied by changing the molecular weight of the PEG used for modification and the number of antibody lysine groups modified. The increase in partition coefficient of HRBC which have been incubated with PEG-modified antibody is directly related to the concentration of antibody used, as seen in the figure that follows.

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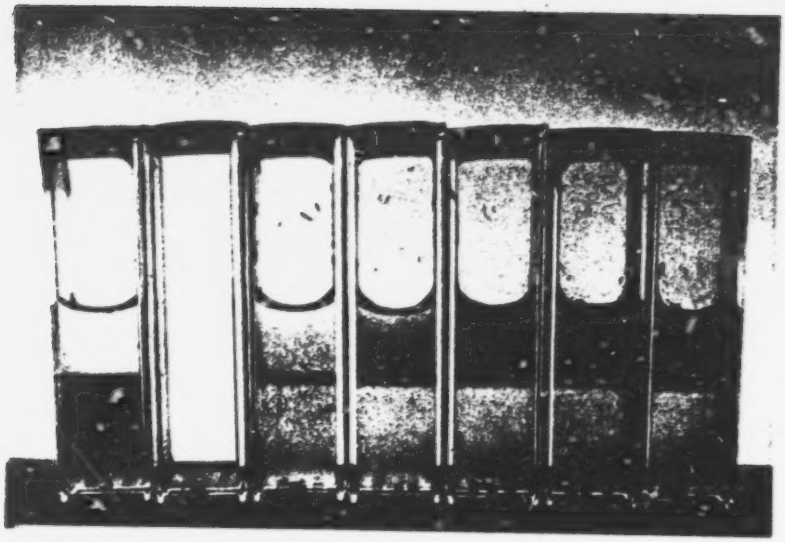

0 8 16 32 64 128

Incubation Concentration of PEG-modified Antibody (ug/ml)

Experiments were performed to demonstrate that one cell population can be selectively separated from a mixture of cells by the use of PEG-modified antibody ligands. Using a two-phase system in which both SRBC and HRBC ordinarily exhibit overlapping CCD profiles, a mixture of the two cell types were completely separated in 30 transfers following a single incubation with the PEG-modified antibody (see Appendix for complete details and references). Having demonstrated that, in this erythrocyte model system, PEG-modified antibodies can be used as effective affinity ligands in phase partitioning, it is important to continue these studies with other cell types of biomedical significance such as T-lymphocyte subsets or megakaryocyte precursors.

RELATED WORK IN PROGRESS

Another possible use for PEG-modified antibody for cell separation techniques is in the area of electrophoresis. In preliminary experiments, varying concentrations of PEG-modified rabbit antibody (directed against HRBC) were incubated with HRBC and their electrophoretic mobilities were measured. As expected from previous work with PEG coatings (1,2,3), the mobilities of HRBC decreased with corresponding increases in incubation concentrations of PEG-modified antibody. Results seem promising and continuing studies may lead to electrophoretic separation of cell populations after the alteration of their surface properties with PEG-modified specific antibody.

Other experiments performed under this contract with affinity phase partitioning have used lectins as ligands. Lectins are proteins, usually of plant origin, which bind specifically to certain sugars (much as antibodies bind to their specific antigen) (4). Many cells have sugar moieties on their surface to which lectins can bind and various separation techniques using lectins, such as differential agglutination, affinity chromatography, and fluorescence activated cell sorting, have been described (5,6,7). Lectins have some advantages over antibodies for use in affinity partitioning. They are less expensive and are readily available in large quantities. Additionally, unlike antibodies, lectins can be easily removed from the cell surface by adding a large excess of the specific sugar to which they bind.

Preliminary experiments have been performed utilizing a PEG-modified lectin, wheat germ agglutinin (WGA), and human peripheral blood lymphocytes. Bourguignon et al (8) reported unequal binding of T and B lymphocytes to WGA which allowed for their separation using a differential agglutination technique. In single tube partition experiments utilizing PEG-modified WGA, the partition coefficients of all lymphocytes were increased over the controls without the lectin. In these experiments, there appeared to be no preferential increase in partitioning of B lymphocytes over that of T lymphocytes, as was hoped. However, the increase noted for all lymphocytes was a positive result and continued experiments are planned with further preparations which vary in the percentage of WGA lysines that are modified with PEG, and using several incubation concentrations.

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APPENDIX

[illegible]-AFFINITY PARTITION OF CELLS IN AQUEOUS POLYMER TWO-PHASE SYSTEMS

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ABSTRACT

Poly(ethylene glycol) (PEG) was covalently coupled to IgG antibody preparations directed against human red blood cells. This modification reduces the tendency of the antibody to agglutinate cells and increases its affinity for the PEG-rich phase in dextran-PEG aqueous two phase systems. These effects are related to the molecular weight of the PEG used for modification and to the number of PEG molecules attached to the antibody.

Exposure of human red blood cells to PEG-modified antibody causes a substantial and specific increase in cell partition into the PEG-rich phase in a number of PEG-dextran aqueous two phase systems. Pertinent phase-system parameters were examined. Following a single incubation with PEG-derivatized antibody, a

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mixture of sheep and human red blood cells was completely separated in 100 minutes by a 30-transfer countercurrent extraction using a two-phase system which normally offers little resolution.

INTRODUCTION

There is considerable need both in biomedical research and in diagnostic medicine for reliable techniques for specifically separating a discrete population of cells from a mixture. The ideal technique should provide a good yield of cells of high purity while cell function is maintained. The present work indicates that immuno-affinity phase partition has the potential for becoming such a technique.

Aqueous solutions of low concentrations of the polymers dextran and poly(ethylene glycol) (PEG) form two-phase systems consisting of a less dense, PEG-rich phase floating on a dextran-rich phase. When buffered and made isotonic by the addition of salts or other low molecular weight solutes, these aqueous polymer two-phase systems can be used for analytical and preparative separation of a wide range of biological substances including cells, organelles, nucleic acids, and proteins.[1-4] Separation results from differential partitioning of substances between the two phases or, in the case of particles, between the liquid-liquid interface and either of the phases. Resolution can be enhanced by the repeated partitioning provided by

countercurrent distribution and countercurrent chromatography.[1-6]

Factors which influence the partition of macromolecules and particles include size, surface charge, and surface hydrophobicity.[1-3] System characteristics such as interfacial tension and salt composition also influence partition and can be altered by changing the type and concentration of polymers and salts.[1-3]

Another method for altering partition is through the use of affinity ligands.[6-12] A ligand that selectively binds to a particle or macromolecule of interest is covalently coupled to one of the polymers (typically PEG). When mixed in a two-phase system, and allowed to settle, the PEG-bound ligand partitions predominately into the phase rich in that polymer, pulling the target material with it.[7-12] These ligands may be specific, such as enzyme inhibitors for enzyme purification,[7] or nonspecific, like dyes for enzyme or nucleic acid purification[8,9] or fatty acid esters for cell purification.[12]

The present article describes how we have refined the technique of phase partitioning to include specific binding of cell surface antigens by antibody ligands attached to PEG. Several steps are required to demonstrate the efficacy of immuno-affinity cell partition: (1) PEG must be attached to the antibody; (2) PEG-derivatized antibody (PEG-Ab) must partition into the PEG-rich phase and it must remain active; (3) the PEG-Ab must pull the cells of interest into the top phase; and

(4) this cell partitioning must be selective for the targeted cells.

We have chosen to examine the partitioning of human and sheep red blood cells (RBC's) as a simple model system. RBC's have been extensively studied in phase partitioning,[1-3] and have the additional advantage of being easily quantified; in addition, native antibodies are commercially available for immuno-affinity partitioning of these RBC's. Also, one aspect of antibody activity can easily be measured by microtiter hemagglutination assay; but it should be noted that PEG-modified antibody may be rendered effectively monovalent, and thus may still be active and capable of binding RBC's when it can no longer agglutinate RBC's. We chose a polymer phase system in which both human and sheep RBC's partitioned in favor of the interface and then used PEG-bound antibodies to the cell membrane of the human RBC to specifically increase the partition of human RBC's into the top, PEG-rich phase. The effects of phase-system parameters and the influence of PEG molecular weight and degree of derivatization were also investigated. In addition, we have examined the usefulness of PEG-antibodies in countercurrent distribution of cells.

MATERIALS AND METHODS

Unless specified otherwise all reagents were ACS grade, or better, quality from commercial sources. Distilled, filtered, 12 megohm/cm water was used throughout.

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Antibody preparations

Rabbit IgG directed against stroma from human RBC's (native antibody) was obtained from Cooper Biomedical, Malvern, Pennsylvania (lot 22196). Fab fragments derived from this same lot were prepared by controlled papain digestion by Jackson Immunoresearch, Avondale, Pennsylvania. An enriched IgG fraction from sheep, also directed against human RBC's, was kindly provided by Dr. D. E. Brooks, University of British Columbia, Vancouver. This fraction, which had been derived from whole serum by ammonium sulfate precipitation, was then further purified by ion exchange chromatography using a DEAE-silica gel column.[13] Both IgG samples agglutinated human RBC's at concentrations less than 0.2 µg/ml but did not agglutinate sheep RBC's.

Modification of antibodies with PEG

In a typical preparation, 12 mg of protein in 0.5 ml 0.05M $Na_2B_4O_7$, pH 9.2, was added to 1.5 ml 0.1M borate buffer. One ml of cyanuric-chloride activated[14,15] PEG 5000 or PEG 900 monomethyl ether in the borate buffer was then added at 4°C, and the mixture was stirred for one hour. Unattached PEG was removed by diafiltration (50 ml Amicon cell, PM-30 membrane, 30,000 molecular weight cut-off) with 10 volumes of 0.05M borate buffer and 0.025M sodium azide to a final volume of two ml. Typically 27 mg of activated PEG 5000, approximately equimolar relative to IgG lysine amino groups (90 per molecule)[16] resulted in about 50% modification of the lysine amino groups; only 70 of

the 90 lysine groups were sensitive to our method of analysis (below). About half this amount of activated PEG (14 mg) gave 30% modification.

Analysis was performed by the Biuret and Habeeb methods as described previously.[14,15] For the latter method (based on reaction of trinitrobenzenesulfonic acid with free lysine groups), we found it necessary to remove unattached PEG and to keep protein concentrations at approximately 0.6 mg/ml in order to obtain accurate and reproducible results. The 0.1M borate buffer was used rather than 4% $NaHCO_3$ called for in the original procedure.

RBC preparation

Blood samples were obtained by venipuncture from healthy individuals and treated with 10mM EDTA as an anticoagulant. Samples were used fresh following brief storage at 4°C. Shortly before use, one ml aliquots were washed four times by suspension in twenty volumes of isotonic buffer (Isoton II, Coulter Electronics, Hialeah, Florida) and centrifugation for 10 min at 1000 x G, followed by removal of the supernatant and the buffy coat.

RBC quantification

Quantification of mixtures of sheep and human RBC's was based on size differences as disclosed by an impedance cell counter (Coulter Electronics, Hialeah, Florida) equipped with a 100 channel size analyzer interfaced with an Apple II+ computer. In some experiments agglutination hindered use of this method,

and visible spectroscopy was used to measure hemoglobin concentrations and thus cell numbers. For this procedure, partitioning samples were centrifuged at 1000 x G for 10 min, the supernatant removed, and 0.4 ml cyanomethemoglobin reagent (Hycel, Houston, Texas) was added to lyse the cells. After centrifugation to remove cell stroma (8800 x G for 10 min), absorbance measurements were taken at 540 nm and compared to the absorbance of a sample, treated identically, containing a known number of cells.

Direct hemagglutination assay

The method for passive hemagglutination described by Amman and Pelger[17] was modified to directly assay the agglutinating ability of both PEG-Ab and native antibody fractions. Human and sheep RBC's (between one and 7 days old) were collected and washed as described above. Packed, washed cells (0.05 ml) were resuspended in 3.0 ml of solution-I (100 ml 0.01M phosphate buffered saline, pH 7.2, 1.6 ml 30% bovine serum albumin, and 100 mg dextrose). This suspension could be kept for up to five days at 4°C. Before testing, a further 1:7 dilution in solution-I was made.

In V-bottom microtiter plates (Cooke Enginnering, Alexandria, Virginia) serial two-fold dilutions (0.05 ml) of each sample to be tested were made using solution II (100 ml 0.01M phosphate buffered saline, pH 7.2, 0.05 ml Tween 80, 1.0 g polyvinylpyrrolidone, and 1.5 ml 30% BSA). Human and sheep RBC's (0.05 ml) were added to each well.

Microtiter plates were placed on an automatic rotator shelf for mixing (10 min) and then incubated on a flat surface overnight at 25°C. Prior to screening, plates were elevated at a 60° angle for 15 min. Positive agglutination resulted in no "run-down" pattern of RBC's. Results were expressed as the minimum concentration of antibody which caused appreciable agglutination.

Preparation of two-phase systems

Polymer phase systems were prepared as described previously[2,3,5] by mixing appropriate weights of the following aqueous stock solutions (all % w/w): 20% dextran T500 (Pharmacia, Piscataway, New Jersey, lot IE32126 or lot HD26066); 50% PEG 8000 (Union Carbide, lot B529-9104 or lot B-739); 20% dextran T40 (Pharmacia, lot FL18974); 0.6M NaCl; and 0.22M Na_2HPO_4, 0.07M NaH_2PO_4, pH 7.2 buffer. Sodium azide was added to a final system concentration of 0.02% to retard bacterial contamination. Concentrations of dextran solutions were determined by polarimetry.[1-3] All other concentrations were determined gravimetrically. After preparation, phase systems were sterilized by filtration through a 0.2 micron filter and allowed to settle at room temperature overnight. The phases were then separated and stored at 4°C but returned to room temperature before use.

Seven well characterized two-phase polymer systems were used this work:[2,5,18] (a) (5,4)I, 5% dextran T500, 4% PEG 8000, 109 mM Na_2HPO_4, 35 mM NaH_2PO_4, pH 7.2 (buffer I); (b) (5,4)II,

5% dextran T500, 4% PEG 8000, 50mM NaCl, 73 mM Na_2HPO_4, 23mM NaH_2PO_4, pH 7.2 (buffer II); (c) (5,3.5)V, 5% dextran T500, 3.5% PEG 8000, 150 mM NaCl, 7.3mM Na_2HPO_4, 2.3mM NaH_2PO_4, pH 7.2 (buffer V); (d) (4.6,3.9)V, 4.6% dextran T500, 3.9% PEG 8000, buffer V; (e)(5,4)V, 5% dextran T500, 4% PEG 8000, buffer V; (f) (7,4)V, 7% dextran T500, 4% PEG 8000, buffer V; (g) (7,5)V, 7% dextran T40, 5% PEG 8000, buffer V.

These systems were chosen to represent a range of interfacial tensions, electrostatic bulk phase potentials, and dextran molecular weight.[1-5,18]

Single tube partition

The ability of various concentrations of PEG-Ab to increase the partition coefficient K (the ratio of cells in the top phase to the remainder of cells) of human RBC's was studied in single-tube experiments. In 12 x 75 mm tubes, 0.5 ml upper phase containing 2×10^7 human RBC's was incubated with 0.2 ml PEG-Ab solution for 15 min at 37°C. The cells were pelleted by centrifugation at 1000 x G for 10 min and the supernatant was removed. After being washed once in fresh upper phase, cells were resuspended in 1.0 ml upper and 1.0 ml lower phase. The suspension was mixed by inversion twenty times and allowed to settle 15 min for equilibration. Mixing was repeated, the system allowed to settle for 15 min and 0.7 ml of top phase was then promptly removed. A portion of this sample (0.2 ml) was used for impedance counting and the remaining 0.5 ml was centrifuged and quantified by spectrophotometric analysis, as

described above.

Inhibition studies

Prior to single tube partitioning, 0.5 ml human RBC's at a concentration of 4 x 10^7/ml in upper phase was first incubated at 37°C for 15 min with 0.2 ml of unmodified Fab. Cells were then washed once in fresh upper phase and resuspended in 0.5 ml upper phase for incubation with PEG-Ab.

Antibody partition

Both PEG-Ab and native antibody preparations (0.2 ml) at a concentration of 2 mg/ml were added to two ml of a (5,4.25)V system to produce a (4.9,3.6)V system. Each tube was mixed twenty times by inversion and allowed to equilibrate for 15 min. After remixing, the systems were centrifuged at 1000 x G for 5 min. An aliquot of the resulting upper phase was mixed with an equal volume of buffer and its absorbance measured at 280 nm. This value was compared to that of the 100%-control tube in which 0.2 ml of the original sample was added to 0.9 ml of upper phase and 1.1 ml buffer.

BSA partition

Comparison of the partition of unmodified bovine serum albumin (BSA) and PEG-modified BSA was carried out in a (5,4)V system as follows: Preparations (0.1 ml) at a concentration of 4 mg/ml were added to 2 ml of phase system. Tubes were mixed by vortexing twice at 10 minute intervals, and were then centrifuged 5 minutes at 200 x G. Aliquots from each phase (0.7 ml) were diluted to 3 ml with PBS and fluorescence excitement

and monitoring were done at 280 nm and 380 nm, respectively, in a Turner spectrofluorometer with the appropriate phase acting as a zero control.

Automated CCD experiments

Countercurrent distribution (CCD) experiments were performed as previously described,[1-3,5] using a 60-chamber Biosheff MK II apparatus (Biochemistry Department, University of Sheffield, Sheffield, England). The lower cavity volume for this machine is 850 µl. Since all runs described in this work consisted of 30 transfers, either duplicate or two different experiments could be run simultaneously, one on either side of the rotor.

Antibody treatment of RBC's consisted of incubating cells or mixtures of cells with PEG-Ab (1.3 mg/ml) at 37°C for 15 minutes in the same proportions as for the single tube partitioning experiments. After incubation, cells were washed once with fresh upper phase and resuspended to a load mix concentration of 2-4 x 10^7 cells per ml of upper phase. For partitioning of cells, 800 µl of lower phase was added to each of the 60 cavities. 900 µl of sample load mix was added to cavities 0,1,30, and 31. 900 µl of upper phase alone was added to the remaining cavities. In every run, a mixing time of 12 seconds and a settling time of 3 minutes was used. Phases were broken by adding 1.0 ml of buffer to each cavity. Cells were quantified as described above.

RESULTS AND DISCUSSION

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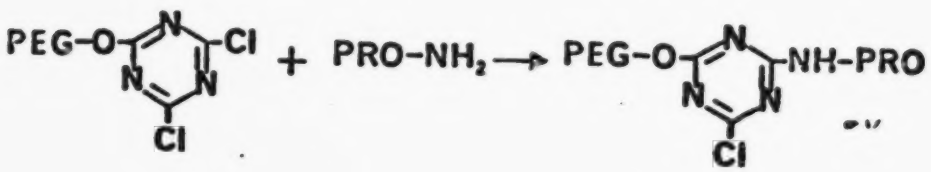
Antibody modification

Of the several methods available for protein-PEG coupling,[19] we chose the method of Abuchowski and Davis[14] in which the PEG is first activated with cyanuric chloride and then coupled to lysine groups on the protein, eq. 1.[15,20]



Using this reaction we could vary the number of lysines substituted with PEG (as shown by reduction in the number of primary lysine amino groups).

The effect of PEG substitution on protein partitioning in a polymer two phase system was first tested with bovine serum albumin (BSA), Table 1. As can be seen from the shifts in BSA partitioning, attaching PEG to a protein can have a dramatic effect on protein partitioning. Similarly, antibody partitioning can also be shifted to favor the top phase, although partitioning shifts are not as dramatic as with BSA, Table 1. Nonetheless, modified antibody was found to strongly favor the top phase, indicating that the proposed affinity partitioning was feasible and that the partition behavior of high MW molecules could be altered by PEG coupling.

Earlier works,[11,20,21] especially those of Abuchowski and Davis[14] have amply demonstrated that PEG-substituted enzymes

remain active although the loss of activity resulting from substitution can be severe with some enzymes and with some coupling methods. The present study is the first to our knowledge utilizing PEG-substituted antibody.[22] As shown in Table 2, PEG-Ab loses some of its ability to agglutinate human RBC's. This effect shows an apparent dependence on PEG MW, the higher MW causing more deactivation. The effect of % substitution on antibody activity is not a simple relationship since the PEG 5000 is more active with a higher % substitution while the PEG 1900 is less active, Table 2. However, measurements of agglutination and %-substitution are sufficiently approximate to make this latter conclusion uncertain. We are presently using more precise techniques to examine the effects of PEG substitution on enzyme activity. For the present study, however, these effects are not of critical importance, since all that is needed is PEG-substituted antibody which is active and partitions to the top phase. In a control experiment PEG-Ab (antibody from sheep) was shown to have no agglutinating activity toward sheep RBC's.

Single tube partition

Next the ability of the PEG-Ab to alter the partitioning of human RBC's was examined. These results are shown in Table 3 and Figures 1 and 2. As we had hoped, the PEG-modified antibody is quite effective at pulling human RBC's into the top phase; the increase in partition varies directly with the amount of PEG-Ab added. Note also that the partitioning shift is observed

with both sheep- and rabbit-derived antibody. Unmodified antibody did not alter RBC partition, either because of extensive agglutination and sedimentation or because PEG is necessary to pull the cells into the PEG-rich phase.

Antibody modified with PEG 5000 gives more dramatic effects, especially evident at the higher concentrations of modified antibody; for example, at 171 µg/ml the PEG-5000 conjugate gave an average % partition of 85 while the PEG-1900 conjugate gave an average % partition of 59. Interestingly, the least ggglutinating PEG-Ab gave the highest % partition; with the more active antibody, agglutination and sedimentation of cell clumps was a problem. Thus it appears that the reduction in antibody agglutinating ability which results upon PEG coupling is advantageous from the standpoint of affinity partitioning.

Finally, it remains to demonstrate that the PEG-Ab is selective in pulling only specific cells into the top phase. Single-tube control experiments clearly showed that partitioning of sheep RBC's was unaffected by PEG-modified antibody. Thus all the components of immuno-affinity phase partitioning of cells are in hand: PEG-modified antibodies will dramatically and selectively shift the partitioning of the targeted cells into the top PEG-rich phase.

In a related study, Sharp et al.[22] have shown that human RBC's bind approximately 15,000 PEG-Ab molecules of rabbit-derived anti-human antibody. This study indicates that the present technique may be applicable to cells having this

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approximate receptor density.

As an additional control experiment, we also examined the ability of monovalent Fab fragments prepared from the same lot of rabbit IgG anti-human RBC (unmodified by PEG attachment) to inhibit the shift in human RBC partitioning that results in the presence of PEG-modified antibody. For this experiment the following concentrations were used: Fab, 686 µg/ml; 47% modified PEG-5000 IgG, 600 µg/ml; and 2×10^7 human RBC's in 2 ml of a (4.6/3.9) phase system. In the absence of Fab 89% of the RBC's partitioned to the top phase. Incubation of the cells with Fab prior to their incubation with PEG-Ab gave a reduction in partitioning to 69% (expected from 150 ug of PEG-Ab). Unfortunately, this experiment was confused somewhat by unexpected cell aggregation by the Fab (which surprisingly, occurred in phase system but not in buffer). Nonetheless, Fab did compete with the PEG-modified IgG in binding with the RBC's and thus gave a reduction in the affinity partitioning effect.

System variation

After these initial experiments, several other phase systems were examined to determine the effects of various phase system parameters on affinity partitioning, Table 4. In brief, we observed that PEG-Ab addition produced the most dramatic shifts in cell partition in systems having a low interfacial tension and a low electrostatic bulk phase potential. As would be expected, increasing the interfacial tension pulled the cells to the interface, making it more difficult to pull the cells into

either phase (e.g., compare the (5,3.5)V, (5,4)V, and (7,4)V systems).

The effects of electrostatic bulk phase potential are difficult to determine since increasing potential (by salt variation) is accompanied by an increase in interfacial tension (e.g., compare the I, II, and V versions of the 5,4 system). Generally, however, it appears that increasing the potential is counterproductive for affinity partitioning since it increases the partition coefficient K (ratio of cells in top to the remainder of cells) in the absence of affinity ligand. Also, the high interfacial tension for high potential systems (e.g., (5,4)I) makes it difficult for the affinity ligand to pull cells away from the interface; note the decrease in partition coefficient in the presence of ligand upon moving from V to II to I versions of the 5,4 system. The net result is that the largest shifts in partitioning from ligand addition are found with the low tension systems such as the (5,3.5)V system.

The final entry in Table 4 is for a (7,5)V system made from dextran of 40,000 molecular weight. The lower dextran molecular weight, as expected,[1,2] gives a lower partition coefficient, with a significant percentage of the cells moving to the lower, dextran-rich phase. This system appears to be of little use for affinity partitioning since the ligand acts to move cells from the bottom phase to the interface, which already contains a large number of the cells. However, it is noteworthy that the PEG-Ab binding is powerful enough to pull the cells from the

bottom phase.

Finally, it should be noted that the (4.6,3.9)V entry (without ligand) for Table 4 is quite different from the same entry for Table 3. These two results differ because different lots of PEG-8000 were used. It is not uncommon in partitioning to observe this sort of unexplained dependence on polymer lots.[18]

Countercurrent distribution

The shifts in partitioning produced by adding PEG-Ab are not large enough to produce total separation in one step. This is a familiar situation in phase partitioning with aqueous polymer two-phase systems, and the usual approach is to utilize an automated device which provides repeated partitioning.[1-6] In the present case we have performed thirty-transfer CCD experiments with a Biosheff MK2 apparatus. In a CCD device the cells are mixed with the two-phase system in one cavity, then the top phase is shifted to the adjacent bottom phase and the bottom phase is shifted to the adjacent top phase, Fig. 3. These affinity CCD experiments could be performed in two ways. Either PEG-Ab could be added to every cavity, in which case a large quantity of PEG-Ab would be required, or the cells could be incubated once with the PEG-Ab prior to CCD; in this latter case little PEG-ligand would be required.

In Figure 4 we have presented the results of three CCD experiments. In the first, Figure 4a, untreated human RBC's

circles) and human RBC's previously incubated with PEG-Ab for 15 minutes at 37°C (triangles) were subjected to separate thirty-transfer CCD runs. As would be expected on the basis of the higher partition coefficient for human RBC's in the presence of PEG-Ab (from single tube experiments), the human RBC's incubated with PEG-Ab gave a peak well separated from that of the untreated human RBC's. Fortunately, the incubation technique provided sufficient exposure of the RBC's to antibody, thus dramatically reducing the amount of PEG-Ab required to perform CCD experiments.

The experiment of Figure 4a was performed with rabbit-derived PEG-Ab with 30% of the lysines substituted by PEG. This particular PEG-Ab sample gave extensive cell agglutination. To avoid cell sedimentation in this case we used a large excess of PEG-Ab in our incubation procedure to lessen the likelihood of a particular antibody encountering more than one cell. Interestingly, not all PEG-Ab preparations gave this same behavior. For example in Figure 4c we show a CCD curve for untreated and treated human RBC's in which incubation was done with a relatively small amount of PEG-Ab in which the IgG was rabbit-derived and had 47% of its lysines substituted with PEG. In this case an antibody excess was not required because cell sedimentation was minimal. The poor separation of Figure 4c results apparently because insufficient PEG-Ab is bound to the human cells. In Figure 4b we show CCD curves for the weakly agglutinating antibody of Figure 4c but at the same

concentration as in Figure 4a; nearly identical results are obtained.

These results emphasize that appreciable variation in PEG-Ab preparations was found. On the negative side, we must say that the origin of this variation is unclear, while on the positive side, we can note that all the variants were effective in immuno-affinity cell partitioning.

Prior to attempts to separate mixtures of human and sheep RBC's, control experiments were done, Figure 5. In these experiments, sheep and human RBC's which were not incubated with PEG-Ab revealed identical CCD behavior, with both RBC's peaking in early fractions.

In our final experiment, shown in Figure 6, sheep and human RBC's were mixed, incubated with PEG-Ab, then subjected to a thirty-transfer CCD run. Again, the human and sheep RBC's were cleanly separated, with the human RBC's moving far down the CCD train.

SUMMARY

In summary, we have demonstrated that immuno-affinity phase partitioning with PEG-modified antibodies as affinity ligands is an effective technique for cell purification in an erythrocyte model system. This advance might reasonably be expected to be applicable to cell separations of significant biomedical interest. Work is in progress in our laboratories to explore this prospect.

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Table 1. Effects of PEG substitution on BSA and native antibody partitioning in dextran/PEG two-phase systems.

protein modified	% of lysines modified	% partition[c]
BSA[a]	0	31 ± 3
BSA	10	89 ± 3
BSA	39	96 ± 1
BSA	47	96 ± 1
BSA	76	98
native-Ab[b]	0	50 ± 3
native-Ab	47	87 ± 3

[a] Bovine serum albumin in a (5,4)V system, dextran T500, PEG 8000; see Materials and Methods. [b] Sheep IgG anti-human RBC's in a (4.6,3.9)V system; dextran T500, PEG 8000; see Materials and Methods. [c] Percentage of total protein partitioning into the upper phase; BSA ($\bar{x}$ ±SD, n = 2); native-Ab ($\bar{x}$ ±SD, n = 4).

Table 2. Effects of PEG substitution on ability of antibody to agglutinate RBC's.

PEG $\bar{M}_w$ [a]	% of lysines modified	minimal hemagglutination concentration (μg/ml)
Control	0	<0.2
A - 5000	27	23 ± 8
B - 5000	40	19 ± 8
C - 5000	51	5 ± 2
D - 1900	45	1 ± 0
E - 1900	70	3 ± 1

[a] Molecular weight of PEG used for substitution of sheep IgG anti-human RBC's. Letter designation refers to Figure 1. [b] Lowest antibody concentration exhibiting appreciable hemagglutination in microtiter assay ($\bar{x} \pm$ SD, n = 3).

Table 3. Partitioning of human RBC's as a function of PEG-Ab concentration and degree of antibody modification.

Modification[a] (%-MW-origin)	[PEG-Ab] (μg/ml)	% partition[b] 5.35	10.7	21.4	42.8	85.7	171
27%-5000-SHE - A		47	46	49	53	79	92
40%-5000-SHE - B		33	39	48	56	77	84
50%-5000-SHE - C		36	38	38	45	54	80
45%-1900-SHE - D		41	34	39	44	55	57
70%-1900-SHE - E		40	45	46	47	54	61
47%-5000-RAB[c]		3.0	3.0	4.0	12	35	80

[a] Given as % lysines modified by PEG; MW of PEG used; and origin (sheep or rabbit) of antibody; A-E designation refers to Figure 1. [b] Duplicate determinations with average standard deviation of 2.4%. Partitioning was done in separate batches of (4.6,3.9)% phase system (dextran T500, PEG 8000; see Materials and Methods). In absence of PEG-Ab 2% partition of human RBC's was observed in the rabbit experiments and 35% in the sheep experiments (see Discussion). [c] Additional values of [PEG-Ab]/% partition: 322/93; 643/90; 1286/84; and 2200/83.

Table 4. Effect of phase system composition on ability of PEG-Ab to induce alteration of RBC partition.

Two-phase system[a]	interfacial tension (μN/m)[b]	electrostatic bulk phase potential (mV)[b]	cell location[c]	% partition[d]	
				no PEG-Ab	PEG-Ab
(5,4)I	11.0	2.2	upper	14 ± 1	58 ± 4
(5,4)II	8	1.0	upper	6 ± 1	64 ± 1
(5,3.5)V	0.7	0.2	upper	2 ± 1	77 ± 1
(4.6,3.9)V	3	0.2	upper	2 ± 1	69 ± 3
(5,4)V	4.9	0.3	upper	0 ± 1	30 ± 1
(7,4)V	17.2	0.2	upper	0 ± 1	4 ± 2
(7,5)V[a]	5.0	0.2	upper	0 ± 1	1 ± 1
			interface	45 ± 3	97 ± 3
			lower	55 ± 3	3 ± 1

[a] All systems compounded with PEG 8000 and dextran T500 of the same lots except (7,5)V which was prepared with dextran T40; see Materials and Methods. [b] Taken from reference 18. [c] Referring to the upper or lower phase or to the interface between them. [d] Mean ± SD of two independent determinations using 643 μg/ml of PEG-Ab (from rabbit).

Figure Legends

Figure 1. Effects of PEG molecular weight, extent of modification, and incubation concentration of PEG-Ab (derived from sheep) on the upper-phase affinity (log K) of human RBC. K is defined as the ratio of the number of cells in the top phase to the remainder. A: PEG 5000, 27% modification; B: PEG 5000, 40% modification; C: PEG 5000, 51% modification; D: PEG 1900, 45% modification; E: PEG 1900, 70% modification.

Figure 2. Effect of incubation concentration of PEG-Ab (derived from rabbit) on the upper-phase affinity (log K) of human RBC.

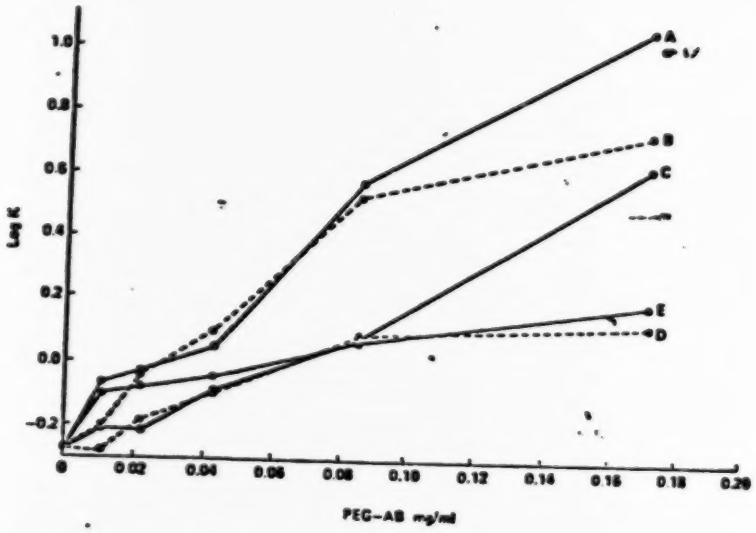
Figure 3. Thin-layer countercurrent distribution (CCD) in aqueous polymer two phase systems. The phases are mixed, allowed to separate, and material distributed between cavities is separated by transferring the upper cavity onto a fresh lower cavity. This process can be repeated (from ref. 5).

Figure 4. Superimposed thirty-transfer CCD runs of human RBC in a (4.6,3.9)V two phase system. A: RBC incubated with 1.3 mg/ml PEG-Ab (30% modification) and washed prior to CCD (Δ); control RBC incubated in buffer only and washed prior

to CCD. B: Identical to A except a different preparation of PEG-Ab used for incubation (47% modification). C: Identical to B except that an incubation concentration of 0.325 mg/ml was used.

Figure 5. Superimposed thirty-transfer CCD runs of human (O) and sheep (□) RBC's in a (4.6,3.9)V two-phase system. Results are averages of four runs for human and seven runs for sheep.

Figure 6. Thirty-transfer CCD of a mixture of 7×10^7 human (O) and sheep (□) RBC's in a (4.6,3.9)V two-phase system following incubation with 1.3 mg/ml PEG-Ab (from rabbit) and washing with phase system to remove unabsorbed antibody.


Log K
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0.8
0.6
0.4
0.2
0.0
-0.2
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
PEG-AB mg/ml
A
B
C
D
E

FIGURE. 1.

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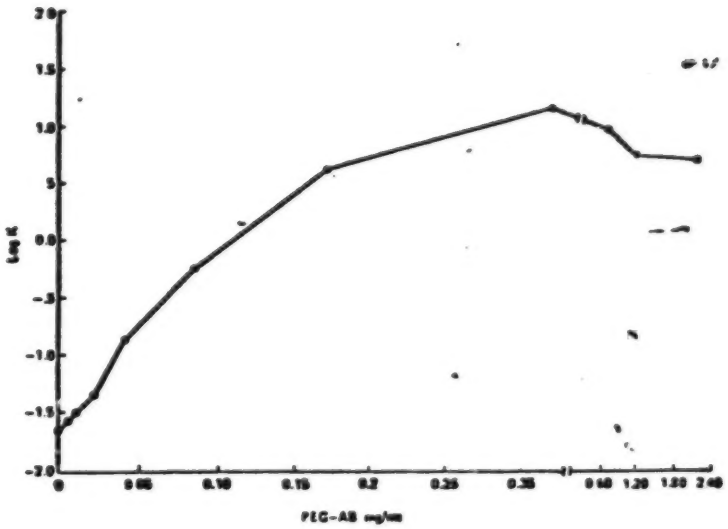

FIGURE 2.

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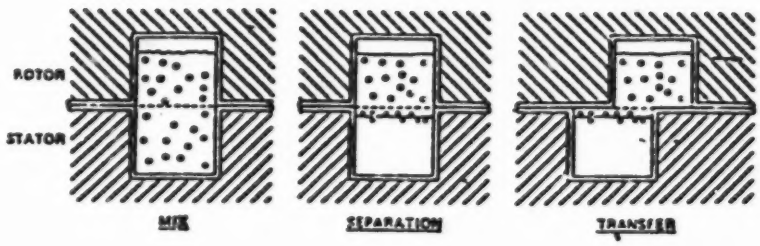

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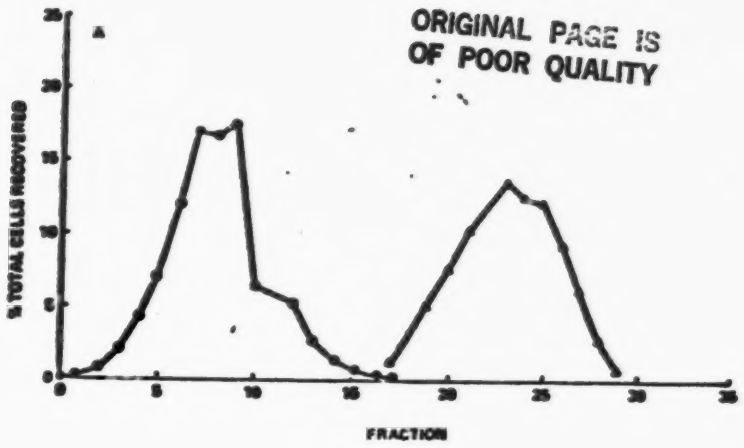


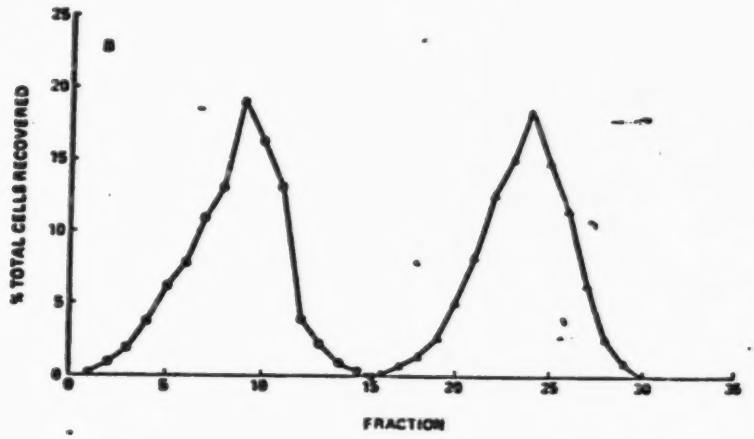
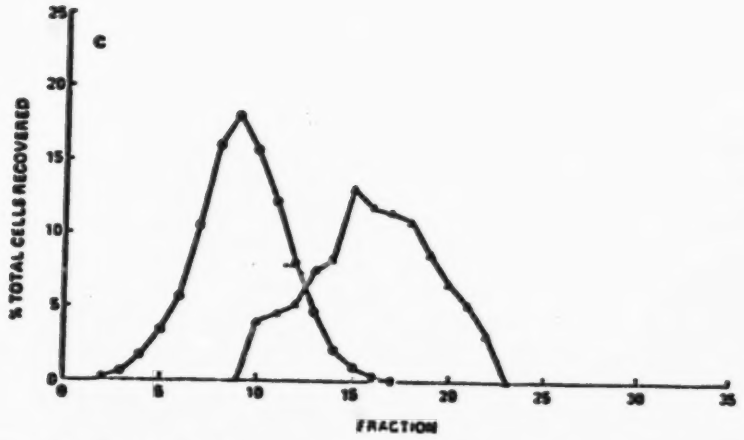


FIGURE 4.



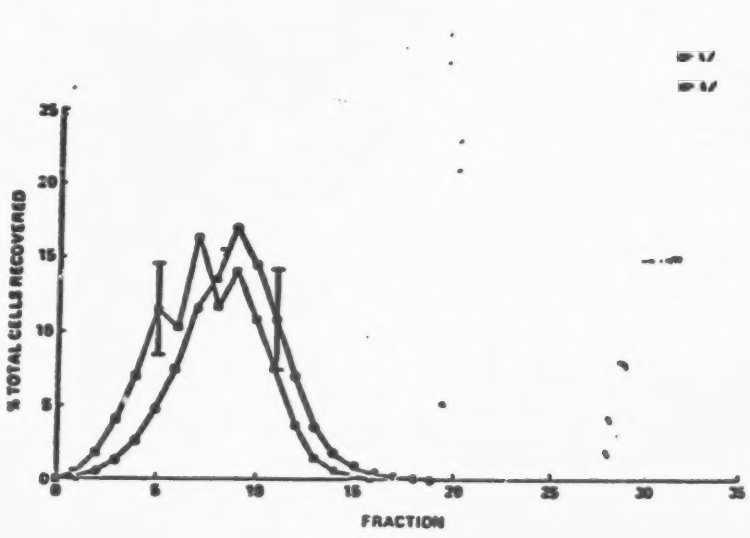


FIGURE 5.

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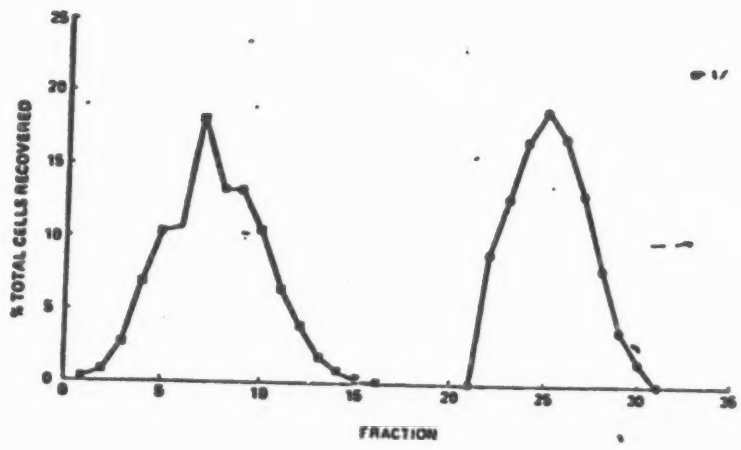

FIGURE 6